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(54) Title: IMMUNOGENIC PREPARATIONS AND VACCINES ON THE BASIS OF RNA

(57) Abstract: The present invention relates to the field of vaccination and immune stimulation by the use of nucleic acids, in particular RNA, coding for one or more antigens of pathogenic micro organisms.

Sequence of βg-HBS-βgαn RNA

(CAP)ggagacaagctagcttgcttgttctttttgcagaagctcagaataaac
gctcaactttggcagatccgaattcatatgtcgacgcgaagcttGGGCCATGC
AGTGGAATTCCACAACCTTCCACCAAACTCTGCAAGATCCCAGAGTGAGAGGC
CTGTATTTCCCTGCTGGTGGCTCCAGTTCAGGAACAGTAAACCCTGTTCTGAC
TACTGCCTCTCCCTTATCGTCAATCTTCTCGAGGATTGGGGACCCTGCGCTGA
ACATGGAGAACATCACATCAGGATTCCTAGGACCCCTTCTCGTGTTACAGGCG
GGGTTTTTCTTGTTGACAAGAATCCTCACAATACCGCAGAGTCTAGACTCGTG
GTGGACTTCTCTCAATTTTCTAGGGGGAACTACCGTGTGTCTTGGCCAAAATT
CGCAGTCCCCAACCTCCAATCACTCACCAACCTCTTGTCCTCCAACTTGTCCT
GGTTATCGCTGGATGTGTCTGCGGCGTTTTATCATCTTCCTCTTCATCCTGCT
GCTATGCCTCATCTTCTTGTTGGTTCTTCTGGACTATCAAGGTATGTTGCCCG
TTTGTCCTCTAATTCCAGGATCCTCAACAACCAGCACGGGACCATGCCGGACC
TGCATGACTACTGCTCAAGGAACCTCTATGTATCCCTCCTGTTGCTGTACCAA
ACCTTCGGACGGAAATTGCACCTGTATTCCCATCCCATCATCCTGGGCTTTCG
GAAAATTCCTATGGGAGTGGGCCTCAGCCCGTTTCTCCTGGCTCAGTTTACTA
GTGCCATTTGTTCAGTGGTTCGTAGGGCTTTCCCCCACTGTTTGGCTTTCAGT
TATATGGATGATGTGGTATTGGGGGCCAAGTCTGTACAGCATCTTGAGTCCCT
TTTTACCGCTGTTACCAATTTTCTTTTGTCTTTGGGTATACATTTAAACCCT
AACAAAACAAAGAGATGGGGTTACTCTCTAAATTTTATGGGTTATGTCAT
TGGATGTTATGGGTCCTTGCCACAAGAACACATCATACAAAAAATCAAAG
AATGTTTTAGAAAACTTCCTATTAACAGGCCTATTGATTGGAAAGTATGT
CAACGAATTGTGGGTCTTTTGGGTTTTGCTGCCCCTTTTACACAATGTGG
TTATCCTGCGTTGATGCCTTTGTAgatctggttaccactaaaccagcctcaag
aacacccgaatggagtctctaagctacataataccaacttacacttacaaaat
gttgtcccccaaaatgtagccattcgtatctgctcctaataaaaagaaagttt
cttcacattctaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaccccccccccc
ccccccccccccccccctgca

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Immunogenic preparations and vaccines on the basis of RNA

The present invention relates to the field of vaccination and immune stimulation by the use of nucleic acids, in particular RNA, coding for one or more antigens of pathogenic micro organisms.

Nucleic acids are novel tools and of growing importance for the prophylactic vaccination and for therapy of infectious diseases or malignant diseases associated with cancer.

Typically, vaccines on the basis of DNA are bacterial plasmids containing genes for tumor antigens or pathogens, which are transcribed after injection into the host organism. In general, this transcription is realized by the use of strong promoters, in particular through viral promoters (Liu, Fu et al. 1998). This type of vaccination offers an alternative to traditional *in vivo* immunisation methods through the reduction of amount of living viruses and the risks associated therewith (Mandl, Aberle et al. 1998) and also to the immunisation with peptides having a limited efficiency due to the genetic variability of the populations (Deres, Schild et al. 1989).

Since RNA is higly susceptible to hydrolysis by ubiquitous ribonucleases, previous approaches of vaccination on the basis of nucleic acids concentrated to the use of DNA. The use of RNA offers in any case advantages in comparison to the use of DNA, in particular in the field of safetyness. The use of RNA *in vivo* does not require promoters derived from viruses, and it does not represent a risk of integration into the genome. On the contrary, the use of DNA may cause an integration into the genome, which may occur by chance or also as a consequence of homologous recombination. In turn, this may lead to the inactivation of cellular

genes but also to a deregulation on the level of their expression. In the worst case, this integration can be the offspring of a tumor. A further source of risk in the vaccination using DNA is the induction of anti-DNA antibodies which may act as potential pathogens, in particular in the case of autoimmune diseases, e.g. in Lupos erythematodes. The administration of RNA, in particular messenger RNA (mRNA), for immunisation therefore *a priori* represents a much more safer approach in comparison to DNA (Lu, Benjamin et al. 1994).

Preliminary results using β-galactosidase as a model antigen for the purpose of examining the value of RNA in anti-tumor vaccination have shown that the injection of RNA causes a specific immune response (Hoerr, Obst et al. 2000).

By experiments carried out with RNA molecules coding for antigens useable in the fields of vaccination and therapy, the inventors have now found that the injection of RNA molecules can effectively lead to an immune response specific for said antigens. Therefore, these results, which are presented in the experimental part below, offer a way for novel perspectives for a vaccination which is safe and which shows an efficiency being independent of the genetic material.

According to the first aspect, the present invention relates to an immunogenic preparation containing mature messenger RNA coding for at least one antigen of a pathogenic agent.

Preferably, the mature mRNA is non-replicative.

The term "mature mRNA" is understood to comprise any RNA molecule containing a coding sequence, which is capped and has a polyA tail. The coding sequence can code for both an antigen containing a protein or a naturally occurring peptide, and a molecule containing at least one non-naturally occurring antigen such as a polyepitope consisting of a sequence of peptides which are naturally distributed over one or more proteins.

According to the present invention, the ribonucleotides which form the mature mRNA may be natural or modified ribonucleotides, in particular in order to introduce an increased resistance against RNases. Different examples of modifications are outlined as follows. As an ex-

ample of a ribonucleotide analogue which may be used for the synthesis of the RNA molecules contained within the preparation according to the present invention, the Sp diasteriomers of the ribonucleosid-5'-O-(1-thiotriphosphates) may be mentioned (Tohda, Chikazumi et al. 1994).

In case the mature mRNA is a so-called naturally occurring molecule, it is present in isolated or purified form when compared to its natural cellular context or to the organism producing said mRNA. In the case of a mature mRNA, a natural molecule is understood to be structurally identical in comparison to a sequence produced in a cell or an organism, in particular in a pathogenic organism. As envisaged above and in the following examples, the mature mRNA according to the present invention can also have a modified sequence when compared with a sequence which is naturally produced in a cell. It can also be a recombinant sequence.

Therefore, the sequence and in particular the functional domains (mainly the coding regions, the cap and the polyA tail) may be derived from different sections of the mature mRNA. In particular, however, they can also be derived from different DNA sequences and/or from different organisms. In this context they are characterised as "heterologous".

The mature mRNAs in the preparation according to the present invention may be obtained by *in vivo* transcription or *in vitro* transcription of a DNA molecule, e.g. as it is described in the protocol of the experimental part below.

However, they can also be produced by any other suitable method.

According to the present invention, the expression "pathogenic agent" designates pathogenic micro organisms or any part thereof such as their toxins from which it are known or which are said to be implicated in the development of diseases in humans and animals, including viruses as well as retroviruses, e.g. HIV retrovirus, or the virus causing Hepatitis A, B or C, the Ebola virus, the Westnile virus, bacteria and parasites, such as plasmodium, as well as unconventional agents such as prions.

In a preferred embodiment, the mature mRNA consists of a coding sequence which is capped and which has a polyA tail.

In particular, the present invention relates to an immunogenic preparation which comprises the above-mentioned RNA in a form which is compatible with a host, in particular a patient in which a protection or protective response against a pathogenic agent has been observed wherein said protection or protective response, respetively, may include, e.g. a prevention in form of a vaccination or a prophylactic or curative treatment after a contamination with the pathogenic agent. This contamination allows to define the RNA which is to be used in the immunogenic preparation.

The expression "protective response against a pathogenic agent" means the protection against a pathogen as well as its direct or indirect effects in the host being infected with the agent.

Here, the term "prophylaxis" means a preventive vaccination while the terms "treatment" and "improvement" relate to an intervention after an infection. "Treatment" means that the administration of a preparation of the present invention causes the clearance of the infectious agents in the patient and his or her cure. In the other case, when the stimulation of the immune response by an immunogenic preparation according to the present invention does not allow the complete cure of the patient, there is just an improvement of his or her clinical state.

Typically, an immunogenic preparation according to the present invention contains at least one of the following constituents: A nucleic acid coding for an antigen of choice and interest, a solvent, e.g. an aqueous solvent, or any solvent which allows to maintain the integrity of the nucleic acid, an adjuvant such as, e.g., aluminium hydroxide, Freund's adjuvant, oligonucleotides having a CpG motif or any other adjuvant known to a person skilled in the art and, finally, any stabilising agent known to a skilled person, such as, e.g. protamine.

The immunogenic preparation according to the present invention is preferably a preparation formulated for constituting a therapeutic preparation. This expression comprises a treatment

or the prevention of a disease following immediately after an infection by the pathogenic agent.

According to a preferred embodiment of the immunogenic preparation of the present invention, the sequence of the mRNA additionally contains sequences being capable of increasing the half-life of the RNA in the cytosol.

"Stabilising sequences" may be derived from any RNA which is known to have a certain stability. They can also be of partial or of complete synthetic nature. As an example of stabilising sequences which are of use in the present invention there may be cited the transcribed but not translated sequences (UTR) of the β-globin gene (human or other), or the consensus sequence of the general formula (C/U)CCANxCCC(U/A)PyxUC(C/U)CC which is contained in the 3' UTR of the highly stable RNA coding for α-globin, α(I)-collagen, 15-lipoxygenase or for tyrosine hydroxylase (Holcik & Liebhaber 1997). N represents any nucleotide, Py represents a pyrimidine (T, U or C) and x means that a nucleotide is repeated one or more times (Nx means that a random nucleotide N occurs x times) (Holcik & Liebhaber 1997). According to the present invention, a combination of these sequences or their association with other sequences is also envisagd.

The above-defined immunogenic preparation containing at least one stabilising sequence which is selected from transcribed but not translated sequences (UTR) of the gene coding for β-globin and the consensus sequence (C/U)CCANxCCC(U/A)PyxUC(C/U)CC contained in the 3' UTR of the RNA coding for α-globin, α-(I)-collagen, 15-liboxygenase and tyrosine hydroxylase, is also subject matter of the present application.

In a further preferred embodiment of the immunogenic preparation according to the present invention, the mRNA comprises a polyA tail of more than 30 adenine residues in the 3' position of its 3' terminus. Preferably, the mRNA does not contain any cytosine residues downstream (i.e. in 3' direction) of said 30 adenine residues.

The above-defined immunogenic preparations wherein the mRNA is synthesized in the presence of modified ribonucleosides in order to improve the resistance with respect to RNases

are integral part of the present invention. In a preferred embodiment of said preparations, the mRNA comprises phosphorothioates.

In contrast to the above-mentioned stabilising sequences, several genes contain sequences which in general destabilise RNA. For example, this is the case for AU-rich sequences which are called AURES and which are found in the 3' UTR sections of numerous instable mRNAs (Caput, Beutler et al. 1986). Therefore, in order to be preferred with respect to their effectiveness, the sequences used for the production of the RNA molecules present in the preparations according to the invention should be altered such that they do not contain any of said destabilising sequences. The same holds for those sites which could be recognised by potential endonucleases, such as the sequence GAACAAG which is contained in the 3' UTR region of the gene coding for the transferin receptor (Binder, Horowitz et al. 1994). These sites can be eliminated from the RNA as it is used in the present invention.

An immunogenic preparation as defined above wherein the mRNA is essentially free from destabilising sequences of the AURES type also falls under the scope of the present invention. Such a preferred mRNA is also free from sequences of the GAACAAG type recognised by endonucleases.

Furthermore, it is possible to improve the initiation and elongation of the translation of the RNA as it is present in the preparation of the invention, for example by mutating codons corresponding to rare tRNAs in that they are substituted by such codons corresponding to abundant tRNAs in mammals. This is also possible by adding sequences such as, e.g., the 5' UTR sequence of the gene coding for HSP 60, which increases the translation of the RNA (Vivinus, Baulande et al., 2001). The object of these modifications is to provide an increased yield of interesting proteins which would increase the effectiveness of the immunisation.

Consequently, the present invention also extends to an immunogenic preparation as defined above, wherein the mRNA additionally contains a sequence which serves to increase the translation rate.

Further subject matter of the present invention is an immunogenic preparation as defined above, which additionally contains at least one RNA stabilising factor, in particular an RNase inhibitor. Such an inhibitor can be, e.g. selected from naturally occurring RNase inhibitors, e.g. Rnasin® (Promega) which is an ubiquitous protein of 460 amino acid residues and which is available in an injectable form for animals (Animal Injectable grade Recombinant Rnasin® (Promega)).

It is further possible to protect the RNA of the immunogenic preparations of the invention in that it is complexed with cationic compounds, preferably polycationic compounds, for example with a cationic or polycationic peptide or protein.

According to a preferred embodiment of the immunogenic preparations, as described in the preceeding paragraph, the RNA-complexing peptide or protein is a protamine, a poly-L-lysine, a poly-L-aginine or a histone.

Furthermore, it is possible to increase the immunogenicity of the preparations of the invention by adding one or more adjuvants.

The term "adjuvant" denotes any chemical or biological compound favouring a specific immune response. This immune response is directed to one or more antigens encoded by the RNA molecules of the preparations of the invention. Depending on the different types of adjuvants various mechanisms can be envisaged. For example, compounds favouring an endocytosis of the RNA by dendritic cells (DC) form a first class of potential adjuvants. Other compounds which allow the maturation of DC, e.g. lipopolysaccharides, TNF-α or the CD 40 ligand, form a further class of potential adjuvants. In general, any immunomodulating agent of the type of "danger signals" (LPS, GP96, oligonucleotides having a CpG motif) or cytokines such as GM-CFS, which allow to increase and/or to influence an immune response against an antigen which is encoded by the injected RNA in a directional manner, can be envisaged as a potential adjuvant.

In particular, an above-defined immunogenic preparation which contains at least one immune system modulating agent selected from lipopolysaccharides (LPS), glycoprotein 96, oligonucleotides having a CpG motif and cytokines, is part of the present invention.

Further subject matter of the present invention is to formulate immunogenic and/or vaccinating preparations having an exactly defined composition in order to avoid any risk of side effects due to an insufficiently identified component. In particular, this is very important in order to avoid pronounced allergic reactions after the injection of the vaccine, which some times occur due to traces of components which where used in the culture medium during the production of the vaccine.

According to the present invention it is possible to provide immunogenic preparations containing a highly purified mature mRNA and one or more also purified adjuvants. Highly purified mRNA denotes an RNA which is treated such that proteins, lipids and DNA fragments are completely eliminated. This can be achieved by carrying out several extraction steps with phenol/chloroform and several precipitations with sodium acetate and ethanol and with lithium chloride. However, it is also possible to achieve the elimination of the above-mentioned components in only a single step in that physical procedures are specifically applied: Columns capable of specifically binding nucleic acids and their separation under defined ionic or pH conditions, or columns of the HPLC type for ion exchange, affinity binding, size exclusion, hydrophobicity, which allow to separate the RNA from other molecules being necessary for the production of the RNA.

Thus, the invention also comprises an immunogenic preparation which, as described above, contains an mRNA which is highly purified before an adjuvant or several adjuvants is/are added.

According to a preferred embodiment of the immunogenic preparations of the invention their composition is formulated for a cutaneous or intradermal administration.

The preparations of this invention are not limited with respect to the nature and number of the antigen of the pathogenic agents which are encoded by the RNA molecules. According to

the present invention, the term "antigen" comprises any molecule containing at least one epitopge. Preferably, an antigen according to the present invention is a molecule capable of triggering an immune response, in particular the formation of antibodies. A preparation according to the present invention may contain only a single type of mRNA having an exactly defined sequence, but it may also contain several different RNAs having different sequences. In the extreme, a preparation of the invention may comprise a whole pool of RNA molecules obtained from a RNA or DNA library, for example from a library obtained from a cell culture of cells infected with a pathogenic agent. In case a preparation of the invention contains mRNA having several or multiple different sequences, the coding sequences of these RNAs may be derived from several different antigens independent of whether they are derived from the same pathogen or not, as well as, on one hand, from one or more antigens and, on the other hand, immune system modulating agent, such as e.g. cytokines.

Therefore, the present invention also relates to an immunogenic preparation as defined above, wherein the RNA represents a library of mRNAs.

Additionally, the preparations according to the present invention may preferably contain multicistronic RNA comprising IRES sequences (IRES = Internal Ribosome Entry Site) which allow, on the basis of the RNA molecule, the production of multiple immunogenic proteins of interest and, in addition, of one or more immunologically active molecules, of cytokines which stimulate or polarise (Th1 or Th2) the immune response.

The immunogenic preparations as described above wherein at least a part or a section of the RNA molecules comprises an internal ribosome entry site (IRES), thus form a further aspect of the invention.

In a preferred embodiment of the preparations according to the present invention comprising an RNA pool and/or wherein at least a part or a section of the RNA molecules comprises an IRES, at least a part or a section of the RNA molecules codes for cytokines which can stimulate or polarise the immune response.

A sequence of the RNA contained in the preparations of the present invention, which codes for at least one antigen of a pathogenic agent, may encode a polyepitope as well as a viral or bacterial, full-length or truncated protein.

One of the possible applications of the preparations according to the present invention is the generation of immunogenic and immune responses against Hepatitis B. In fact, 5% to 15% of adults do not respond to any of the presently used recombinant vaccines. This failure of immune response which is attributed to the HLA type could be overcome by vaccination on the basis of RNA. Additionally, an immune therapy by vaccination with RNA of the 350 million chronic carriers of HBV could be envisaged. The present inventors have shown that the surface antigen of the virus responsible for Hepatitis B (HBsAg) is a good candidate in order to trigger a response against this virus (Loirat, Lemonnier et al. 2000).

Therefore, an immunogenic preparation according to the present invention wherein at least a part or a section of the RNA molecules codes for a surface protein of the Hepatitis B virus is a preferred embodiment of the present invention. In this embodiment of the invention at least a part or section of the RNA molecules may code for the small, medium, and large envelope proteins of the Hepatitis B virus.

In a preferred embodiment of the immunogenic preparations described in the preceeding paragraph, at least a part or a section of the RNA molecules comprise the sequence βg-HBs-βgαn as shown in Fig. 1.

Another target for the immunogenic preparations according to the present invention is the humane immune deficiency virus (HIV). Therefore, in a preferred embodiment of the invention, at least a part or a section of the RNA molecules code for at least one antigen of HIV and, in a preferred mode of the invention, for a polyepitope of HIV. In particular, in this embodiment of the present invention at least a part a section of the RNA molecules may comprise the sequence βg-HIV-HBS-βgαn shown in Fig. 2. In this example, the polyepitope connected to HBS comprises 13 HLA-A2 epitopes derived from different proteins of HIV-1. It can also be envisaged that a number of more important epitopes are connected to HBS,

which are represented by MHC class I proteins and which are derived from numerous proteins of the HIV virus.

The Hepatitis C virus is a further pathogen against which it is desirable to develop a vaccine or at least an agent capable of stimulating an immune response in a host. In this regard, the use of RNA encoding the CORE protein of the Hepatitis C virus (HCV) appears to be of interest. In fact, the CORE protein is the most conserved of the genotypes of the HCV. Additionally, patients infected by HCS show a cellular response against the CORE protein and it is associated with a favourable response to a therapy with interferons. The use of RNA encoding non-structural and structural proteins of the virus could prove to be efficient, such as in the embodiment wherein a part or a section of the RNA codes for the CORE protein on one hand and for other proteins on the other hand.

In another preferred embodiment of the immunogenic preparations according to the present invention thus codes a part or section of the RNA molecules for the CORE protein of the Hepatitis C virus.

The present invention also relates to the use of an immunogenic preparation as defined above for the preparation of a pharmaceutical composition for the prophylaxis, treatment or improvement of an infection with a pathogenic agent, in particular HIV, Hepatitis B virus, Hepatitis C virus, Rous-Sarcoma virus, the parasites responsible for Malaria or the bacteria causing pneumonia and optionally associated with the acceleration or onset of a disease associated with an increased liability to cardio-vascular disorders at it is the case, e.g. with *Chlamydia pneumoniae*.

Another particular important aspect of the present invention is a vaccine characterised in that it comprises any of the above-defined components contained in the immunogenic preparations as described in the whole preceeding text, and which allows to generate a protection against a pathogenic agent in a host (human or animal).

In particular, a vaccine according to the present invention is characterised in that the protection is obtained by an activation of cytotoxic $CT8^+$ T lymphocytes and/or CD4-specific T

helper cells by at least a part or a section of the RNA molecules of the administered preparation and/or by activation of B lymphocytes as antibody producers. The anti-HBs antibodies form the basis of a prophylaxes against an infection with HBV. The T cell response (helper and cytotoxic T cells) is required in order to control an established infection.

The following non-limiting Examples and Figures further illustrate the present invention and demonstrate certain advantages and features thereof.

Figure legends

Fig. 1: Sequence of injected βg-HBS-βgαn RNA

(Uraciles are denoted by the letter T)

The sequence in capital letters codes for the HBS (S2.S) antigen

The start and termination codons for translation are shown in boxes.

The transcribed but not translated regions (5' and 3' UTR of β-globin) are shown in bold.

The sequences in small letters and normal font have no special significance for the vaccination with RNA and are derived from plasmids.

The poly-A-poly-C tail is shown in italic.

The region of the restriction site used for linearisation of the plasmid before transcription is underlined.

Fig. 2 :Sequence of injected βg-HIV-HBS-βgαn RNA

(Uracile is denoted by the letter T)

The sequence in capital letters codes for the polyepitope HLA-A*0201 HIV-HBS (capital letters in bold correspond to the HIV region of the polyepitope HLA-A*0201).

The start and termination codons for translation are shown in boxes.

The transcribed but not translated regions (5' and 3' UTR of β-globin) are printed in small letters and in bold.

Sequences in small letters and normal font have no special significance for the vaccination with RNA and are derived from plasmids.

The poly-A-poly-C tail is shown in italic.
The region of the restriction site used for the linearisation of the plasmid before transcription is underlined.

Fig. 3: Experiment for the restimulation of CTL *in vitro*

BalB/c mice were sacrificed 8 weeks after injection of 20 µg of RNA or DNA in both ears, or of 50 µg RNA into the two rear paws after treatment with cardiotoxins. The spleenocytes were restimulated *in vitro* with an HBS H-2d epitope and tested in a cytotoxicity experiment against transfected targets expressing the surface antigen HBs (P815/S) and P815 cells as a negative control.

The specific cytotoxic activity was determined by measuring the short-time release of ^{51}Cr. After incubation for 4 h at 37°C, the supernatants were collected and counted with a β-counter. For each curve, the vertical axis represents the percentage of lysed target cells (or percentage of cytotoxicity) with respect to a control lysis of 0% and a control lysis of 100%. The percentage of cytotoxicity = 100 x (cpm[target cells + CTL] - cpm[target cells alone]) / (cpm[target cells alone + detergent]-cpm[target cells alone]), and the horizontal axis indicates the ratio of effector/target cells. Each curve corresponds to the spleenocytes of a single animal.

Fig. 4: IFN-γ (Fig. 4 A) and IL-4 (Fig. 4 B) ELISPOTS

ELISPOT tests were carried out with spleenocytes obtained as in the experiment as described in Fig. 3 (spleenocytes obtained at the same time point as described in the experiment according to Fig. 3 but without restimulation of T cells *in vitro*).

The ordinate represents the number of IFN-γ (Fig. 4 A) and IL-4 (Fig. 4B), respectively, producing cells, per 10^6 cells.

On the abscissa are represented the different immunisation conditions of the mice, and the antigen used for the stimulation is indicated by the filling of the corresponding column.

Fig. 5: Specific ELISA for examining the humoral anti-Hepatitis B response

Two months after the injection of DNA or RNA the blood of the mice was collected by retrobulbar punctation utilising heparinised glass pipettes and the sera recovered by centrifugation were tested for the presence of anti-HBs and anti-preS2 antibodies by a specific ELISA. Purified recombinant particles containing the small protein S of HBV (1 μg/ml) or the synthetic peptide preS2 (120-145) (1 μg/ml) were used as the solid phase. After blocking with PBST supplemented with 10% fetal calf serum serial dilutions of serum were added. After extensive washing, bound antibodies were detected with anti-mouse immunoglobulins (total IgG) conjugated with horseradish peroxidase (Amersham, UK). Antibody titers were determined by the method of limited dilution. The sera of the mice were tested individually and the titers are expressed as mean values of at least three determinations. Dilutions below 1/100 where considered negative. Anti-HBs titers are expressed as geometric group average +/- standard deviation (GMT +/- SEM) of values of individual animals which in turn are the mean value of duplicate or triplicate experiments.

Fig. 6: ELISPOTS with fresh spleenocytes

HHD mice were injected with 20 μg DNA, RNA or RNA in combination with protamine in each ear (intra ear, i.e.), or with 50 μg RNA in each rear paw after treatment with cardiotoxin (i.m.). Three weeks thereafter, spleenocytes were restimulated *in vitro* with peptides corresponding to the invidual epitopes and the immune response was determined by measuring the released IFN-γ using ELISPOT.

The figure shows the average number of spots obtained for each peptide with 5 mice.

This experiment was carried out by immunizing the mice with a sequence shown in Fig. 2.

The DNA or RNA constructs injected into the mice and serving for immunisation code for the HIV-1 epitopes A9M-Y/I9V-Y/K9L8S-Y/T9V-Y/V9L-Y/P10 L-V11V-Y/P9L-S9L-Y/E9V-L10V-L9V-Y/K9L-HBS protein.

Examples

Use of mRNA for obtaining cellular and humoral responses against antigens of viral origin

1 - Structure of RNA suitable for vaccination

The RNAs used in the following examples contain transcribed but not translated 5' and 3' sequences (5' or 3' UTR) of the RNA coding for β-globin of *Xenopus*. These sequences probably form secondary structures which are recognized by cytosolic proteins. They allow an increase of the half-life of the RNA in the cytosol.

However, it is also possible that the RNA effective for vaccination does not contain said stabilising sequences.

Additionally, the stabilising sequences of β-globin may be substituted by stabilising sequences derived from another RNA having a long half-life in the cytosol. For example, the consensus sequence (C/U)CCANxCC(U/A)PyxUC(C/U)CC contained in the 3' UTR of the highly stable RNAs coding for α-globin, α(I)-collagene, 15-lipoxygenase and tyrosine hydroxylase (Holcik & Liebhaber 1997) may substitute or be added to the 3' UTR sequence of β-globin used in the RNAs described below.

Furthermore, the 3' polyA tail which improves the stability and the translation of the RNA may be elongated and may be free of cytosine residues. RNAs having more than 100 A residues in 3' position and no C residue downstream are preferably used. Furthermore, the in-

crease of the overall number of C or G residues contained in the RNA in general (without leading to modifications in the amino acid sequence of the proteins encoded by the RNA) is also a method for the stabilisation of mRNAs and is also envisaged for improving the efficiency of the immunisation with RNA. A further measure to stabilise the RNA against RNases is to synthesise the RNA in the presence of modified ribonucleotides such as Sp diasteriomers of 5'-O-(1-thiotriphosphate)ribonucleosides. The RNA containing phosphorothioates shows an improved resistance against the degradation by RNases compared to natural RNA, and shows an improved translation into proteins (Tohda, Chikazumi et al. 1994). Their use for the formulation of a vaccine is therefore envisaged.

As mentioned before, it is also possible to eliminate certain sequences which are known to destabilise the RNA.

Finally, it is also envisaged to use multicistronic RNA containing IRES (internal ribosome entry site) sequences which, on the basis of an RNA molecule, permit the production of multiple immunogenic proteins of interest or, in addition, of one or more immunogenic molecules, of cytokins capable of stimulating or polarising (Th1 or Th2) the immune response.

2 - Adjuvants and additives

The inventors have observed that a very careful precipitation of the RNA used for the immunisation of mice (in addition to precipitation steps with lithium chloride) could lead to a decreased efficiency of the immunisation.

For this reason, the co-injection of the vaccine RNA together with immune system modulating agants such as the "danger signals" (e.g. LPS, GP96, oligonucleotides having CpG motives) or cytokines (e.g. GM-CFS) are therefore also envisaged for permitting an increase and/or control of the immune response against the antigen encoded by the injected RNA.

The use of cationic compounds (proteins and others) such as protamine for condensating and protecting the RNA against the degradation by RNases is potentially advantageous, however, according to the results shown herein below, not required.

3 - Antigens suitable for vaccination with RNA

The experiments described herein below illustrate two possible modes for carrying out the invention with respect to the nucleotide sequence introduced into the constructs and coding for an antigen. These two modes are on one hand a sequence coding for a polyepitope (in the present case an HIV polyepitope) and on the other hand the sequence coding for a whole protein (here, an envelop protein of HBV).

The potential targets of the vaccination by RNA injection are *a priori* not limited. As selected examples for antigens which are suitable for this type of vaccination there can be mentioned the antigens of Hepatitis A, Hepatitis B and Hepatitis C viruses, HIV retrovirus, *Chlamydia pneumoniae*, Rous-Sarcoma virus or the parasites responsible for Malaria (*Falsiparum sp.*).

For the development of an RNA vaccine against Hepatitis C virus, the use of an RNA appears to be interesting, which codes for the CORE protein of HCV. In fact, the CORE protein of this virus is the most conserved of the genotypes of HCV. Additionally, patients infected with HCV show a cellular response against the CORE protein and it is associated with a favourable response to a therapy with interferons. However, the use of RNA coding for non-structural or structural proteins could also prove to be effective.

4 - Production of RNA

The sequence of the βg-HBS-βgαn RNA is shown in Fig. 1.

The sequence of the βg- HIV-HBS-βgαn RNA is shown in Fig. 2.

The sequences printed in bold small letters are the 5' and 3' sequences which are transcribed but not translated (5' or 3' UTR) of the RNA coding for *Xenopus* β-globin.

The sequences coding for the molecules HBS S2.S and HIV-polyepitope-HBS, respectively, were derived from the plasmid pCMV HB-S2.S-d1 as described by Marie-Luise MICHEL

(Michel, Davis et al. 1995) and from the plasmid pCMV-B10 described by Husseyin FIRAT (Firat, Garcia-Pons et al. 1999), respectively. Both fragments contain a *Hin* dIII site upstream of the gene and an *Nsi* I site (rendered free enzymatically) downstream. They were ligated into a plasmid allowing the production of RNA (this plasmid contained a promoter recognized by SP6 RNA polymerase as well as 5' and 3' non-translated sequences of the RNA encoding β-globin of *Xenopus*, separated by a multiple cloning site), which itself is digested by the enzymes *Hind* III and *Bgl* II (rendered free enzymatically). The resulting plasmid construct is linearised with *Pst* I, subsequently freed from any proteins by extractions with phenol/chloroform and by ethanol and ammonium acetate precipitations, and subsequently suspended in RNase-free water. This DNA was transcribed using the SP6 mMESSAGEmMACHINE® kit from Ambion. This kit is serves to produce RNA molecules, the majority of which (80%) contain a CAP structure ($m_7G(5')ppp(5')G$) in the 5' position. After DNAse treatment of the transcription reaction the RNA is precipitated with lithium chloride, resuspended in water and quantified by measuring the optical density at 260 nm. Subsequently, the preparation is purified from traces of protein by extraction with lithium chloride and then by precipitation with ethanol and ammonium acetate, before it is resuspended in RNase-free water at 10 μg/ml. After incubation overnight by 4°C, the RNA is diluted using RNase-free Hepes buffer (150 mM NaCl; 10 mM Hepes pH 7.5) to 1 μg/μl.

5 - HbsAg expression vectors, HBs RNA and immunisation

The pCMV-S2.S plasmid contains a segment of the region of the genome of HBV coding for the envelope. In this plasmid (Michel, Davis et al. 1995), the preS2 and S regions are placed under the transcriptional control of the immediate-early promoter of the human cytomegalovirus (CMV-i.e.) allowing the expression of the small and medium envelope proteins of HBV, both of which contain the surface antigen HBs (HBsAg).

The plasmid DNA used for the immunisation *in vivo* was prepared using DNA purification columns from Qiagen (Endofree Plasmid Kit, Qiagen, Hilden, Germany).

For immunisation based on DNA the expression vector for HBsAg, pCMV-S2.S, was directly injected into the muscle of the anterior tibia for boost injections as described previously, or

directly into the ear pinna. 50 µl DNA at 1 mg/ml were injected into each tibia for a total quantity of 100 µg DNA per mouse. 20 µl DNA at 1 mg/ml were injected into both ears for a total quantity of 40 µg DNA per mouse.

Concerning the immunisation on the basis of RNA, RNA coding for HBsAg was obtained by using a vector containing a fragment sub-cloned from a plasmid pCMV-S2.S, and the SP6 polymerase. The RNA was produced following the method described in the patent application EP 1 083 232. In some experiments the RNA was stabilised using protamine at a ratio of 1/1 g. 20 µl RNA at a concentration of 1 mg/ml were injected behind each ear of BALB/c mice for a total amount of 40 µg RNA per mouse.

For immunisation, groups of 4 to 6 feemale BALB/c mice of 6 to 8 weeks of age were used.

6 - Induction of CTL

The spleens where taken 52 days after immunisation and the spleenocytes were counted and analysed in order to determine the relative proportion of T and B cells. The percentage of $CD8^+$ and $CD4^+$ cells was determined by a FACS analysis of fresh spleenocytes using a direct stain with anti-mouse $CD8^+$ FITC and $CD4^+$ PE antibodies (Pharmingen, San Diego, CA).

7 - Analysis of lymphocyte populations in the spleen after vaccination

The ratio of $CD8^+$ T cells and $CD4^+$ T cells in the groups was comparable. However, the injection into the ear appears to lead to a higher number of B cells (see the following Table 1).

Table 1: Analysis of lymphocyte populations in the spleen after vaccination.

Group	Spleenocytes	$CD4^+$ T cells	$CD8^+$ T cells	CD4/CD8	B cells
pCMVS2 DNA/i.m. (4 mice)	91×10^6 $\pm 31 \times 10^6$	19.8×10^6 $\pm 6.7 \times 10^6$	13.3×10^6 $\pm 2.0 \times 10^6$	1.5	49.5×10^6 $\pm 17.4 \times 10^6$
pCMVS2S DNA/ear (4 mice)	124×10^6 $\pm 22 \times 10^6$	21×10^6 $\pm 3.9 \times 10^6$	10.7×10^6	2	76.3×10^6 $\pm 13.4 \times 10^6$
HBsm RNA ear (6 mice)	131×10^6 $\pm 19 \times 10^6$	26.4×10^6 $\pm 4.0 \times 10^6$	15.9×10^6	1.7	71.5×10^6 $\pm 11 \times 10^6$
HBs RNA + protamine ear (6 mice)	117×10^6 $\pm 17 \times 10^6$	21.8×10^6 $\pm 3.0 \times 10^6$	15.6×10^6 $\pm 4.5 \times 10^6$	1.4	71.8×10^6 $\pm 12.9 \times 10^6$

8 - Measurement of CTL activity

The spleens of the mice was recovered 8 weeks after immunisation with RNA or DNA. The spleenocytes where cultured in α-MEM medium (Gibco) (10×10^6 cells per well of a 24-well-plate), supplemented with 10 mM Hepes, non-essential amino acids, sodium pyruvate (1 mM), β-mercaptoethanol (50 nM) and 10% fetal calf serum. These cells were stimulated over 5 h with 1 μg/ml of the peptide derived from S (amino acids 28-39 counted from the first methionin of the small envelope protein) corresponding to the epitope restricted to $H\text{-}2L^d$. The effector cells were used in a cytotoxicity experiment carried out after 7 days of culture. The cytolytic activity of the cells was tested in short-term ^{51}Cr release experiments against transfected targets expressing HBsAg (P815/S) and P815 cells as negative control. After 4 h incubation at 37°C, the supernatants were collected and counted using a β-counter. The spontaneous and maximum releases were determined in wells containing either medium alone or lysis medium (5% Triton, 1% SDS). The specific lysis was calculated as follows: (experi-

mental release - spontaneous release) / (maximum release - spontaneous release) x 100 for each experiment. The specific lysis was calculated for each data point in duplicate. The CTL-activity was determined for each mouse at different ratios of effector/target cells (E/T).

9 - Study of cytotoxic T-lymphocytes after vaccination (Fig. 3)

For the detection of CTL, the spleenocytes were activated *in vitro* over 7 days with the Ld-restricted peptide S-28-39, then tested for their lytic activity against P815/S-cells transfected with HBs. Non-transfected P815 cells where used as controls. The results are presented in Fig. 3. The cytotoxic responses were determined in each group of immunised mice. The intramuscular injection of DNA into the treated muscle gave the highest lysis rate of the target cells. Cytotoxic T cells were found in 4/6 mice after injection of RNA together with protamine, while 5/5 and 6/6 mice had cytotoxic T cells in the spleen after injection of DNA or naked RNA, respectively.

This indicates that the injection of HBs-RNA, naked or associated with protamine, is capable of activating cytotoxic T cells *in vivo*, which recognize the HBs antigen prepared in an endogenous manner.

10 - Quantification of HBs-specific T cells producing IFN-γ

Interferon-γ (IFN-γ) releasing cells were quantified by an immune spot assay specific for a given cytokine (enzyme-linked immunospot or "ELISPOT") after stimulation with peptides or with the HBs antigen. ELISA plates having a planar nitrocellulose-coated bottom were covered with 50 µl of rat anti-IFN-γ murine antibodies (5 µg/ml, Pharmingen, San Diego, CA) over night 4°C and then saturated with RPMI 1640 containing 10% fetal calf serum for 2 h at 37°C. Spleenocytes (1 x 10^6/well of a 96-well plate), which were produced as described above for the CTL experiments, were incubated in complete α-MEM medium at 37°C in the presence of 5% CO_2 using different antigens for stimulation. The cells were incubated with HBV-S peptides (3 µg/ml) or HBsAg particles (1 µg/ml). Two peptides derived from S restricted to class I (28-39 = I-12-L and the peptide 15 = S362-372 W-11-L) and a peptide derived from preS2 restricted to the MHC class II (preS 138-165) were used for activation. The

background was evaluated with cells in medium. The cells were suspended by buffering the wells, then lysed with water. After washing with PBS containing 0.05% Tween 20, a biotinylated rat anti-IFN-γ murine antibody (1 μg/ml, Pharmingen, San Diego, CA) was added for an incubation of 90 minutes at room temperature. The wells were washed as before, then incubated for 1 h 30 min with an alkaline phosphatase-streptavidine conjugate (Boehringer-Mannheim, Germany) at a dilution of 1:1000 in PBS. Subsequently, a solution of 2.3 mM of 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) and of Nitroblue-Tetrazolium (NBT) (Promega, Madison, WI) diluted an alkaline buffer solution was added. When the spots became visible, the reaction was stopped with water and air-dried. The number of IFN-γ secreting spots was counted using an ELISPOT-reader (Zeiss) and the results were expressed for each group as the mean value of IFN-γ secreting cells per 10^6 spleen cells.

Two months after immunisation, the HBs-specific T cells in the spleen of the mice immunised with DNA injected only intra-muscularly were detected (Fig. 4A). These T cells produced IFN-γ after activation with a peptide restricted to class I, derived from S, or the peptide restricted to class II, derived from pre-S2. In contrast thereto, no spot could be detected after activation of the spleenocytes from mice injected with DNA or with RNA into the ear. This leads to the conclusion that the activated T cells were not presented in the spleen of the mice two months after immunisation using RNA or DNA administrated over the ear. However, the detection of cytotoxic T cells after restimulation *in vitro* with a peptide (see Fig. 3) indicates that T memory cells were induced by the use of either DNA or RNA using both immunisation pathways.

Comparable results were obtained by using an IL-4 ELISPOT experiment (Fig. 4 B). Few spots corresponded to activated T cells secreting IL-4 after immunisation with DNA (intra muscular) and recognising both the class I peptide derived from S and the class II peptide derived from preS2. The larger proportion of IFN-γ secreting T cells indicates that the T helper response was oriented to a Th-1 profile.

11 - Humoral response

Two months after injection of DNA or RNA, the blood of the mice was collected by retrobulbar punctation using heparinised glass pipettes, and the sera, recovered by centrifugation, were tested with respect to the presence of anti-HBs and anti-preS2-antibodies by specific ELISA tests. Purified recombinant particles containing the small S protein of HBV (1 μg/ml) or the synthetic peptide preS2 (120-145) (1 μg/ml) were used as the solid phase. After blocking with PBST (phosphate-buffered saline containing 0.1% of Tween 20), supplemented with 10% fetal calf serum, serial dilutions of serum were added. After extensive washing, bound antibodies were detected by the use of anti-mouse immunoglobulins (total IgG) labelled with horseradish peroxidase (Amersham, UK). The antibody titers were determined by the method of limited dilution. The sera of the mice were tested individually and the titers were the mean value of at least 3 determinations. The dilutions below 1/100 were considered negative. The anti-HBs titers are expressed as geometric group average ± standard deviation (GMT ± SEM) of values of individual animals which were in turn the mean values of duplicate or triplicate experiments.

The antibodies specific for HBsAg or for the preS2-domain of the medium protein of HBV were detected by ELISA in the serum of 4 of 4 mice 8 weeks after intramuscular injection of pCMV-S2.2 (Fig. 5). Neither naked RNA nor RNA in combination with protamine led to a significant production of anti-HBs and anti-preS2 antibodies in the mice injected by the intra auricular pathway. In contrast thereto, weak titers of anti-HBs antibodies were detected in the serum of 2/4 mice injected with DNA over the intra auricular pathway. These results suggest that DNA probably persists longer than RNA, allowing a constant production of the HBs antigen.

12 - HIV-polyepitopes-HBS expression vector, HIV-polyepitops-HBS RNA and immunisation

The plasmid pCMV-B10 contains a polyepitope comprising 13 HLA-A2-epitopes of HIV-1. In this plasmid (Firat, Garcia-Pons et al. 1999), the polyepitope sequence is placed under the

transcriptional control of the immediate-early promoter of the human cytomegalovirus (CMV-i.e.).

The plasmid DNA used for immunisation *in vivo* was prepared by the use of DNA purification columns from Qiagen (Endofree Plasmid Kit, Qiagen, Hilden, Germany).

For immunisation on the basis of DNA, the vector for expression of the polyepitope, pCMV-B10, was directly injected into the muscles of the anterior tibia for boost injections as described previously, or directly into the ear pinna. 50 µl DNA at 1 mg/ml were injected into each tibia for a total quantity of 100 mg DNA per mouse. 20 µl DNA at 1 mg/ml were injected into both ears for a total quantity of 40 µg DNA per mouse.

Concerning the immunisation on the basis of RNA, the RNA coding for the polyepitope was obtained by the use of a vector containing a sub-cloned fragment of the pCMV-B10 plasmid, and the SP6 polymerase. The RNA was produced following the method described in the patent application EP 1 083 232. In some experiments, the RNA was stabilised by the use of protamine at a ration of 1/1 g. 20 µl RNA were injected behind each ear of BALB/C mice for a total amount of 40 µg RNA per mouse.

For immunisation, groups of 4 to 6 female BALB/c mice of 6 to 8 weeks of age were used.

13 - Quantification of T cells specific for a HIV epitope and producing IFN-γ

The mice were sacrificed three weeks after injection of RNA.

IFN-γ releasing cells were quantified by an ELISPOT as described above after stimulation with the 13 HLA-A2 epitopes encoded by the pCMV-B10 plasmid. ELISA plates having a planar nitrocellulose-coated bottom (Multiscreen, Millipore, Molsheim, France) were covered with 50 µl of rat anti-IFN-γ murine antibodies (5 µg/ml, Pharmingen, San Diego, CA) over night at 4°C, then saturated over 2 hours at 37°C with RPMI 1640 containing 10% fetal calf serum. Spleenocytes (1 x 10^6/well of a 96-well plate), obtained as described above in the experiments with respect to activated CTL, were incubated for 40 h in complete α-MEM me-

dium at 37°C in the presence of 5% CO_2 using different antigenic stimulations. The cells were incubated separately with each of the HIV epitopes encoded by the pCMV-B10 plasmid, wherein said epitopes were present at a concentration of 10 μM. The background was evaluated with cells in medium. The cells were suspended by buffering the wells, then lysed with water. After washing with PBS containing 0.05% Tween 20, a biotinylated rat anti-IFN-γ murine antibody (1 μg/ml, Pharmigen, San Diego, CA) was added for an incubation of 90 min at room temperature. The wells were washed as described above, before an incubation for 1 h 30 min with an alkaline phosphortase-streptavidine conjugate (Boehringen, Mannheim, Germany) at a dilution of 1:1000 in PBS. Then, a 2.3 mM solution of 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) and of Nitroblue Tetrazolium (NBT) (Promega, Madison, WI), diluted in an alkaline buffer solution, was added. When the spots became visible, the reaction was stopped with water and air-dried. The number of IFN-γ secreting spots was counted using an ELISPOT-reader (Zeiss), and the results were expressed for each group as the mean value of IFN-γ secreting cells per 10^6 spleen cells.

The results are shown in Fig. 6.

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Claims

1. An immunogenic preparation comprising mature mRNA coding for at least one antigen of a pathogenic agent.

2. The immunogenic preparation according to claim 1 wherein said RNA is a non-replicative mature mRNA.

3. The immunogenic preparation according to claim 1 or 2 wherein the sequence of the RNA comprises additionally stabilising sequences capable of increasing the half-life of the RNA in the cytosol.

4. The immunogenic preparation according to claim 3 wherein at least a part of the stabilising sequences is selected from transcribed but not translated sequences (UTR) of the β-globin gene and the consensus sequence (C/U)CCANxCCC(U/A)PyxUC(C/U)CC contained in the 3' UTR of the RNAs coding for α-globin, α-(I)-collagen, 15-lipoxygenase and tyrosine hydroxylase.

5. The immunogenic preparation according to any one of claims 1 to 4 wherein the RNA contains at its 3' terminus a polyA tail having more than 30 A residues in the 3' position.

6. The immunogenic preparation according to any one of claims 1 to 5 wherein the RNA has been synthesised in the presence of ribonucleosides modified for resisting the degradation by RNases.

7. The immunogenic preparation according to claim 6 wherein the RNA contains phosphorothioates.

8. The immunogenic preparation according to any one of claims 1 to 7 wherein the RNA is essentially free of destabilising AURES sequences and of sequences recognized by endonucleases.

9. The immunogenic preparation according to any one of claims 1 to 8 wherein the RNA contains additionally a sequence susceptible of increasing its translation.

10. The immunogenic preparation according to any one of claims 1 to 9 comprising at least one RNA stabilising factor, in particular an RNase inhibitor.

11. The immunogenic preparation according to any one of claims 1 to 10 wherein the RNA is complexed with at least one cationic, preferably polycationic, peptide or protein.

12. The immunogenic preparation according to claim 11 wherein the peptide or protein is a protamine, a poly-L-lysine, a poly-L-arginine or a histone.

13. The immunogenic preparation according to any one of claims 1 to 12 comprising one or more adjuvant(s).

14. The immunogenic preparation according to claim 13 comprising at least one immunomodulating agent selected from lipopolysaccharides (LPS), glycoprotein 96, oligonucleotids containing CpG motives and cytokins.

15. The immunogenic preparation according to claim 13 or 14 wherein the RNA is highly purified before the addition of the one or more adjuvant(s).

16. The immunogenic preparation according to any one of claims 1 to 15 wherein its composition is formulated for a cutaneous or intradermal administration.

17. The immunogenic preparation according to any one of claims 1 to 16 wherein the RNA is a pool of mRNAs.

18. The immunogenic preparation according to any one of claims 1 to 17 wherein at least a part of the RNA molecules comprises an internal ribosome entry site (IRES).

19. The immunogenic preparation according to claim 17 or 18 wherein at least a part of the RNA molecules codes for cytokins capable of stimulating or polarising the immune response.

20. The immunogenic preparation according to any one of claims 1 to 19 wherein the sequence encoding at least one antigen of a pathogenic agent codes for a polyepitope.

21. The immunogenic preparation according to any one of claims 1 to 19 wherein the RNA sequence coding at least one antigen of a pathogenic agent codes for at least one full-length or truncated viral or bacterial protein.

22. The immunogenic preparation according to claim 21 wherein at least a part of the RNA molecules codes for a surface antigen of Hepatitis B virus.

23. The immunogenic preparation according to claim 22 wherein at least a part of the RNA molecules codes for the small and medium envelope proteins of the Hepatitis B virus.

24. The immunogenic preparation according to claim 23 wherein at least a part of the RNA molecules has the sequence shown in Fig. 1.

25. The immunogenic preparation according to claim 20 wherein at least a part of RNA molecules code for a polyepitope of HIV.

26. The immunogenic preparation according to claim 25 wherein at least a part of RNA molecules has the sequence shown in Fig. 2.

27. The immunogenic preparation according to claim 21 wherein at least a part of the RNA molecules codes for the CORE protein of Hepatitis C virus.

28. Use of an immunogenic preparation according to any one of claims 1 to 27 for the preparation of a pharmaceutical composition for the prophylaxis, treatment or improvement of the state of a patient after an infection with HIV, Hepatitis B virus, Hepatitis C virus, Rous-Sarcoma virus, the parasite responsible for Malaria, or *Chlamydia pneumoniae*.

29. A vaccine characterized in that it comprises an immunogenic preparation according to any one of claims 1 to 27, allowing the obtention of a protection against a pathogenic agent in a human or animal host.

30. The vaccine according to claim 29 characterized in that the protection is obtained by an activation of cytotoxic and/or helper T lymphocytes and of B lymphocytes specific for at least one antigen encoded by at least a part of the RNA molecules of the preparation.

Fig. 1

Sequence of βg-HBS-βgαn RNA

(CAP)ggagacaagctagcttgcttgttcttttgcagaagctcagaataaac
gctcaactttggcagatccgaattcatatgtcgacgcgaagcttGGGCCATGC
AGTGGAATTCCACAACCTTCCACCAAACTCTGCAAGATCCCAGAGTGAGAGGC
CTGTATTTCCCTGCTGGTGGCTCCAGTTCAGGAACAGTAAACCCTGTTCTGAC
TACTGCCTCTCCCTTATCGTCAATCTTCTCGAGGATTGGGGACCCTGCGCTGA
ACATGGAGAACATCACATCAGGATTCCTAGGACCCCTTCTCGTGTTACAGGCG
GGGTTTTTCTTGTTGACAAGAATCCTCACAATACCGCAGAGTCTAGACTCGTG
GTGGACTTCTCTCAATTTTCTAGGGGGAACTACCGTGTGTCTTGGCCAAAATT
CGCAGTCCCCAACCTCCAATCACTCACCAACCTCTTGTCCTCCAACTTGTCCT
GGTTATCGCTGGATGTGTCTGCGGCGTTTTATCATCTTCCTCTTCATCCTGCT
GCTATGCCTCATCTTCTTGTTGGTTCTTCTGGACTATCAAGGTATGTTGCCCG
TTTGTCCTCTAATTCCAGGATCCTCAACAACCAGCACGGGACCATGCCGGACC
TGCATGACTACTGCTCAAGGAACCTCTATGTATCCCTCCTGTTGCTGTACCAA
ACCTTCGGACGGAAATTGCACCTGTATTCCCATCCCATCATCCTGGGCTTTCG
GAAAATTCCTATGGGAGTGGGCCTCAGCCCGTTTCTCCTGGCTCAGTTTACTA
GTGCCATTTGTTCAGTGGTTCGTAGGGCTTTCCCCCACTGTTTGGCTTTCAGT
TATATGGATGATGTGGTATTGGGGGCCAAGTCTGTACAGCATCTTGAGTCCCT
TTTTACCGCTGTTACCAATTTTCTTTTGTCTTTGGGTATACATTTAAACCCT
AACAAAACAAAGAGATGGGGTTACTCTCTAAATTTTATGGGTTATGTCAT
TGGATGTTATGGGTCCTTGCCACAAGAACACATCATACAAAAAATCAAAG
AATGTTTTAGAAAACTTCCTATTAACAGGCCTATTGATTGGAAAGTATGT
CAACGAATTGTGGGTCTTTTGGGTTTTGCTGCCCCTTTTACACAATGTGG
TTATCCTGCGTTGATGCCTTTGTAgatctggttaccactaaaccagcctcaag
aacacccgaatggagtctctaagctacataataccaacttacacttacaaaat
gttgtcccccaaaatgtagccattcgtatctgctcctaataaaaagaaagttt
cttcacattctaaaaaaaaaaaaaaaaaaaaaaaaaaaaaccccccccccccc
ccccccccccccccccccctgca

Fig. 2

Sequence of βg-HIV-HBS-βgαn RNA

(CAP)ggagacaagctagcttgcttgttcttttttgcagaagctcagaataaac
gctcaactttggcagatccgaattcatatgtcgacgcgaagcttCAGGCCATG
CAGTGGAATTCAGCACTTGTGGAAATCTGTACCGAAATGTACTTAAAAGAACC
AGTTCACGGGGTGTACCTCACGCCGCTCTGCGTTTCTCTTTACCTCAACGCAT
GGGTGAAAGTTGTATACATTTACCAGTATATGGACGACCTTTATCTCACCTTT
GGATGGTGCTTCAAACTGGTGCTCGACGTGGGGGACGCGTACTTTTCCGTGTA
TCTGGTCAAACTATGGTACCAATTAAGTCTTTACAACACCGTCGCGACCCTTT
ATATGATGACAGCATGCCAAGGCGTTCTTCTTGACACCGGCGCAGACGACACG
GTGCTCCTGTGGAAGGGCGAGGGAGCAGTTTATCTTACACCACTTTGTGTTAC
TCTCGAGCATATAGGACCAGGGAGAGCATTTGTGGTACCTCTAGAAGAAGCTT
GGGATCCGTCGAGGATTGGGGACCCTGCGCTGAACATGGAGAACATCACATCA
GGATTCCTAGGACCCCTTCTCGTGTTACAGGCGGGGTTTTTCTTGTTGACAAG
AATCCTCACAATACCGCAGAGTCTAGACTCGTGGTGGACTTCTCTCAATTTTC
TAGGGGGAACTACCGTGTGTCTTGGCCAAAATTCGCAGTCCCCAACCTCCAAT
CACTCACCAACCTCTTGTCCTCCAACTTGTCCTGGTTATCGCTGGATGTGTCT
GCGGCGTTTTATCATCTTCCTCTTCATCCTGCTGCTATGCCTCATCTTCTTGT
TGGTTCTTCTGGACTATCAAGGTATGTTGCCCGTTTGTCCTCTAATTCCAGGA
TCCTCAACAACCAGCACGGGACCATGCCGGACCTGCATGACTACTGCTCAAGG
AACCTCTATGTATCCCTCCTGTTGCTGTACCAAACCTTCGGACGGAAATTGCA
CCTGTATTCCCATCCCATCATCCTGGGCTTTCGGAAAATTCCTATGGGAGTGG
GCCTCAGCCCGTTTCTCCTGGCTCAGTTTACTAGTGCCATTTGTTCAGTGGTT
CGTAGGGCTTTCCCCCACTGTTTGGCTTTCAGTTATATGGATGATGTGGTATT
GGGGGCCAAGTCTGTACAGCATCTTGAGTCCCTTTTTACCGCTGTTACCAATT
TTCTTTTGTCTTTGGGTATACATTTAAACCCT
AACAAAACAAAGAGATGGGGTTACTCTCTAAATTTTATGGGTTATGTCAT
TGGATGTTATGGGTCCTTGCCACAAGAACACATCATACAAAAAATCAAAG
AATGTTTTAGAAAACTTCCTATTAACAGGCCTATTGATTGGAAAGTATGT
CAACGAATTGTGGGTCTTTTGGGTTTTGCTGCCCCTTTTACACAATGTGG
TTATCCTGCGTTGATGCCTTTGTAgatctggttaccactaaaccagcctcaag
aacacccgaatggagtctctaagctacataataccaacttacacttacaaaat
gttgtcccccaaaatgtagccattcgtatctgctcctaataaaaagaaagttt
cttcacattctaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaccccccccccccc
ccccccccccccccccccctgca

Fig. 3

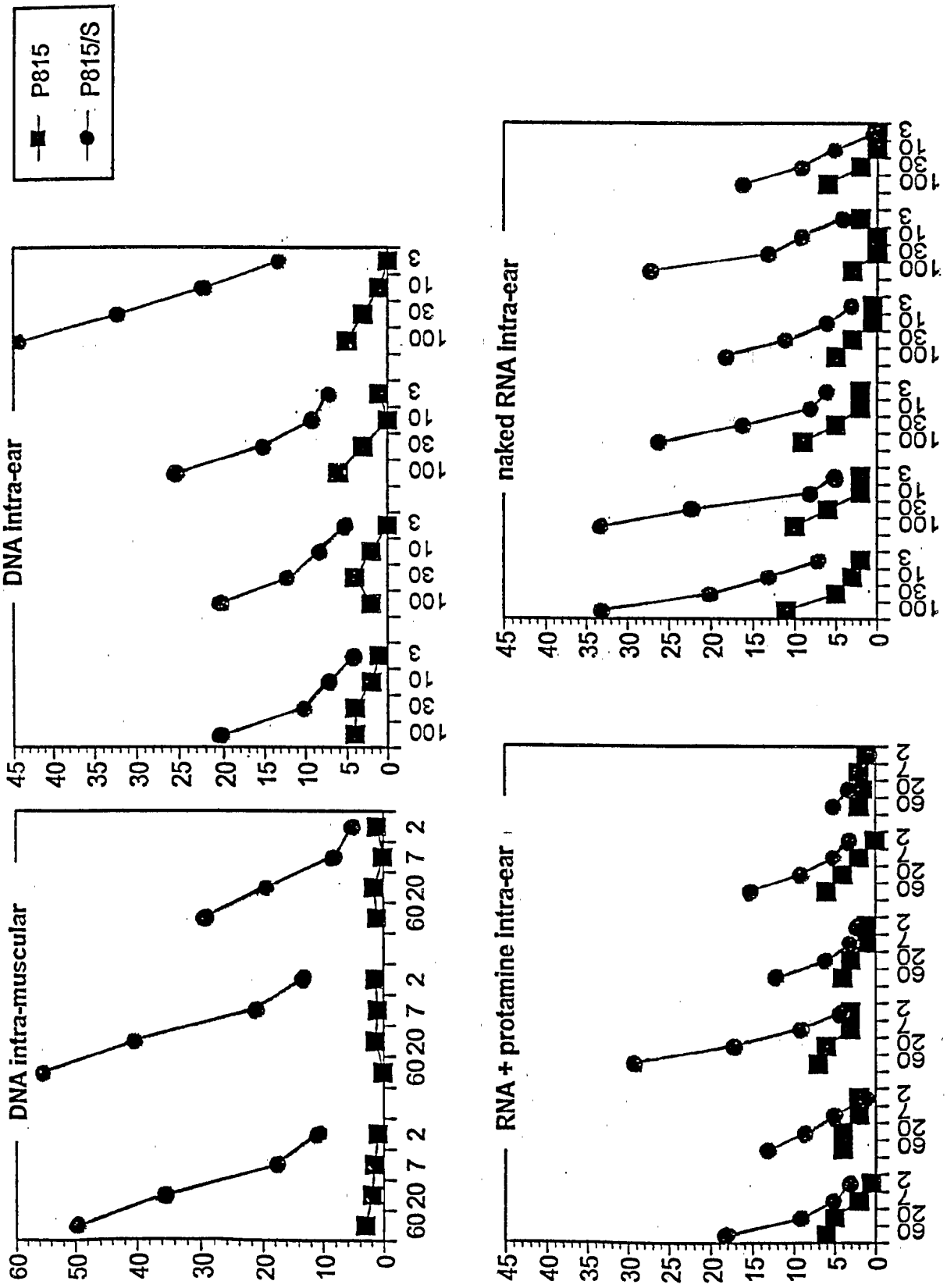
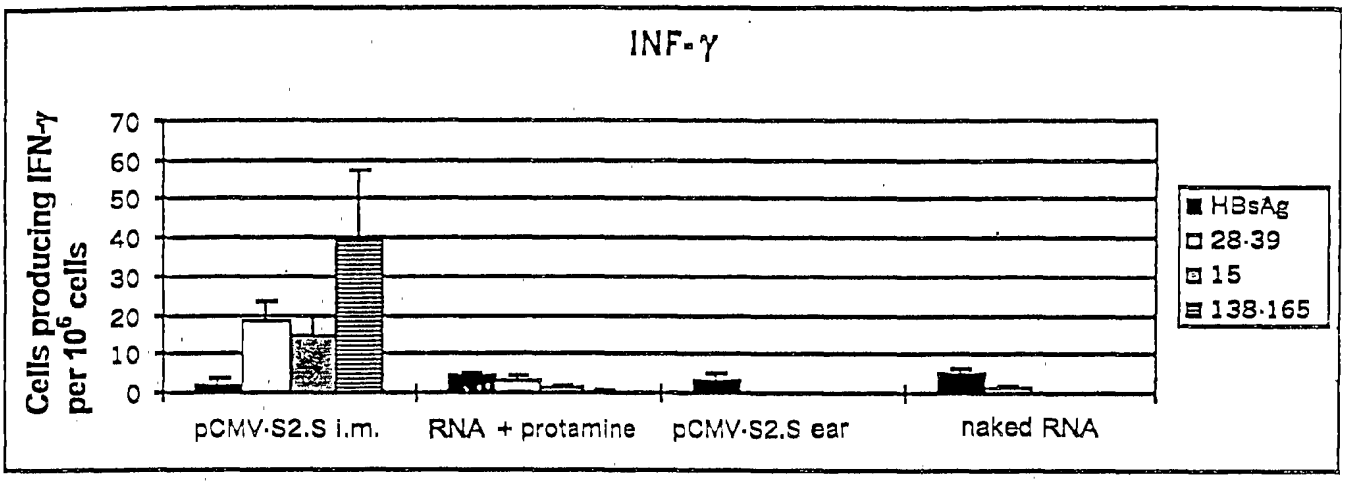

P815
P815/S
DNA intra-ear
DNA intra-muscular
naked RNA intra-ear
RNA + protamine intra-ear

Fig. 4

A


INF-γ
Cells producing IFN-γ per 10^6 cells
70
60
50
40
30
20
10
0
pCMV-S2.S i.m.
RNA + protamine
pCMV-S2.S ear
naked RNA
HBsAg
28-39
15
138-165

B

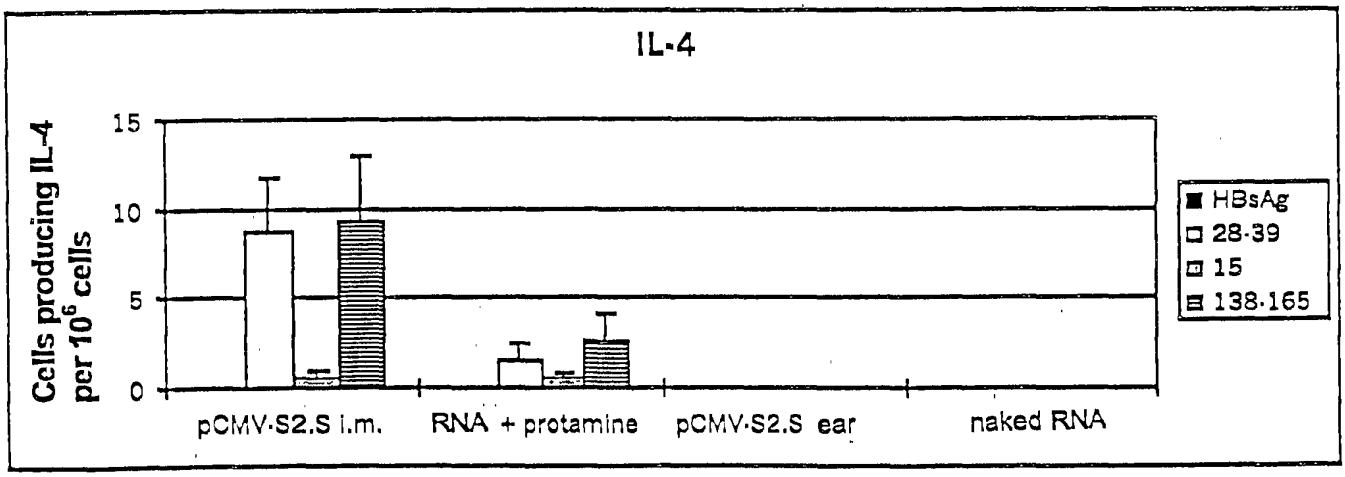
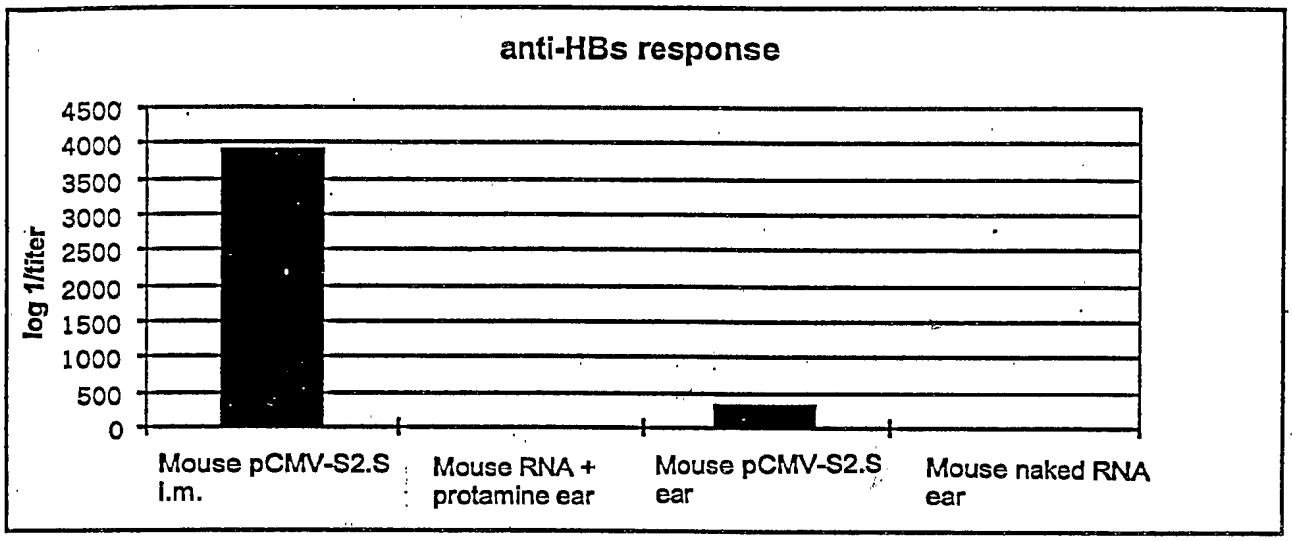

IL-4
Cells producing IL-4 per 10^6 cells
15
10
5
0
pCMV-S2.S i.m.
RNA + protamine
pCMV-S2.S ear
naked RNA
HBsAg
28-39
15
138-165

Fig. 5

A


anti-HBs response
log 1/titer
4500
4000
3500
3000
2500
2000
1500
1000
500
0
Mouse pCMV-S2.S i.m.
Mouse RNA + protamine ear
Mouse pCMV-S2.S ear
Mouse naked RNA ear

B

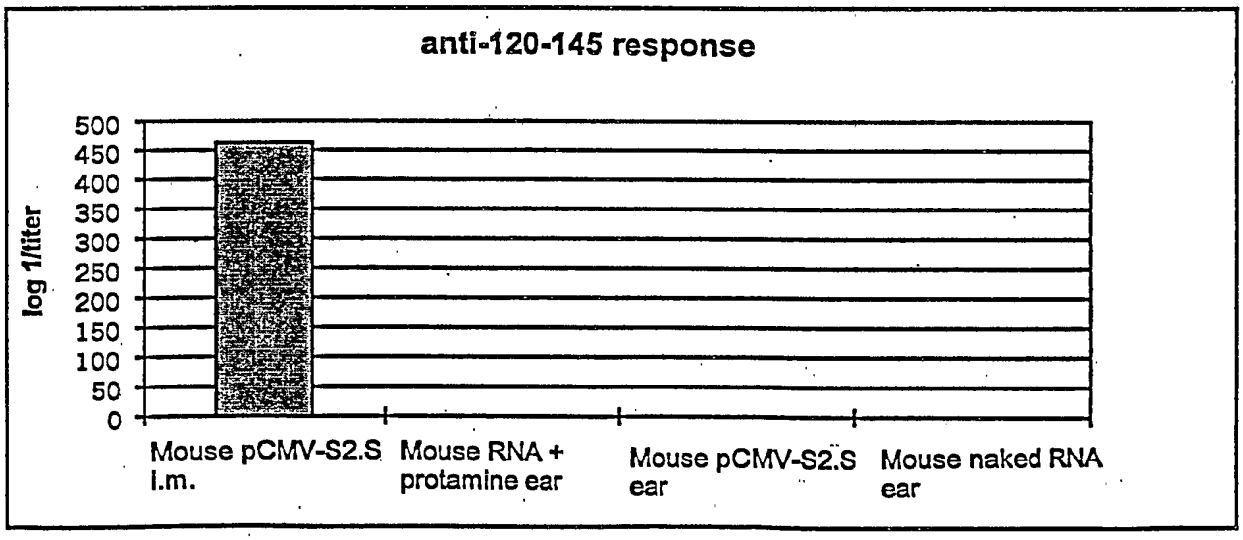
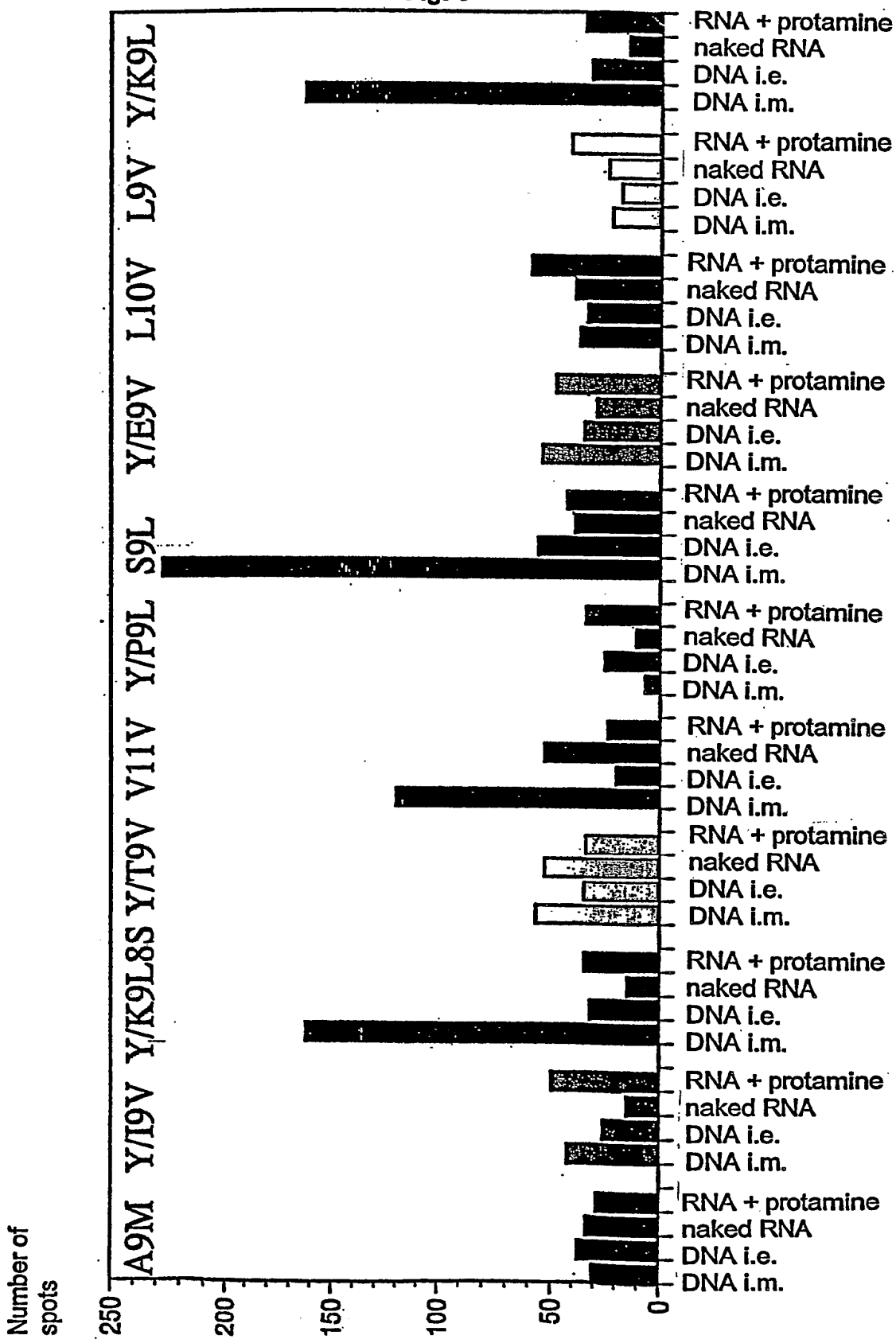

anti-120-145 response
log 1/titer
500
450
400
350
300
250
200
150
100
50
0
Mouse pCMV-S2.S i.m.
Mouse RNA + protamine ear
Mouse pCMV-S2.S ear
Mouse naked RNA ear

Fig. 6


Number of spots
250
200
150
100
50
0
A9M
Y/I9V
Y/K9L8S
Y/T9V
V11V
Y/P9L
S9L
Y/E9V
L10V
L9V
Y/K9L
RNA + protamine
naked RNA
DNA i.e.
DNA i.m.